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13. ABSTRACT (Maximum 200 Words)

Breast cancer development typified by the overexpression of growth factors and growth factor receptors, expression of cell cycle markers such as cyclin D1 and c-myc, expression of chemokines such as RANTES, and development of resistance to cancer therapies. We and others have provided evidence that the transcription factor NF-kappaB and associated activities are expressed/activated in human breast cancer. Specifically we found that the NF-kB2/p52 NF-kappaB subunit and Bcl-3 are expressed in a significant number of breast tumors. Our preliminary data indicate that Bcl-3 expressing cells exhibit upregulation of cyclin D1, c-myc, and the chemokine RANTES and that it leads to the strong suppression of the ability of Taxol to induce cell death. Our goals are to: (i) identify genes regulated by Bcl-3 that may be relavent to the progression of the disease, (ii) determine the mechanism whereby Bcl-3 blocks cancer-therapy induce apoptosis, and (iii) determine if Bcl-3 and the associated NF-kappaB subunit p52 are required for the development of experimental breast tumors in animal models. These goals may provide significant new insight into breast cancer progression and treatment.

14. SUBJECT TERMS
NF-kappaB, breast cancer therapy, suppression of apoptosis, Chemoresistance, Her-2?neu

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INTRODUCTION:

Breast cancer development and progression is characterized by dysregulation of growth factors, growth factor receptors, expression of cycle cell markers including cyclin D1 and c-myc, expression of chemokines such as RANTES, and development of resistance to cancer therapies. We and others have provided evidence that NF-κB activity is activated in breast cancer cell lines and in a majority of human breast tumors (for example, see Cogswell et al., 2000; Romieu-Mourez et al., 2003). Recently, Hung and colleagues provided evidence that the kinase that regulates NF-κB (IKKβ) is active in breast cancer and is involved in the inactivation of a pro-apoptotic transcription factor Foxo3a (Hu et al., 2004). Our analysis in human breast tumor tissues is that p52/NF-κB2 (a non-classic form of NF-κB) and Bcl-3, an IκB homologue, are upregulated in a number of breast tumors (Cogswell et al., 2000). Additionally, it was reported that Bcl-3 is upregulated in breast cancer cells following withdrawal of estrogen, leading to growth and hormone independence (Pratt et al., 2003). These findings relate to our previous publication (Westerheide et al., 2000) that Bcl-3 can directly regulate cyclin D1 gene expression (which is known to be upregulated in a majority of breast tumors). Our goals are to understand the biological roles of the NF-κB regulatory system in breast cancer as related to development of this disease as well as in controlling cancer therapy resistance.

BODY:

The Specific Aims/Statement of Work indicate the following priorities: (i) identify genes and regulatory processes regulated by Bcl-3, (ii) determine mechanisms associated with the ability of Bcl-3 to alter responses to chemotherapies and radiation, and (iii) determine if Bcl-3 and/or p52 are required for the development of experimental breast tumors using animal models.

In year one we had proposed to begin to identify genes and regulatory processes controlled by Bcl-3 and induced by Her-2/Neu and to begin analysis of Bcl-3 and Her-2/Neu in breast tumors. In the initial year of this Department of Defense breast cancer grant, we have made significant progress towards determining molecular mechanisms associated with the function of Bcl-3. We generated MCF-7 cells that overexpress Bcl-3 and found the following: (i) these cells are highly resistant to UV-induced cell death and (ii) these cells exhibit a blunted p53 response. This data is shown in Appendix I as Figures 1 and 2. These data suggest that breast cancer cells that express Bcl-3 will be resistant to the induction of apoptosis, at least partly through the ability to suppress p53 function. Correspondingly, we analyzed Bcl-3 null cells, and found that Bcl-3 expression is required for UV-induced p53 suppression. This mechanism appears to require the ability of Bcl-3 to induce Mdm2 function (see Fig. 3A and 3B, in Appendix I). Thus, the ability of Bcl-3 to suppress p53 induction may involve the Bcl-3 dependent activation of Mdm2 gene expression. Consistent with the ability of Bcl-3 to suppress apoptosis, Bcl-3 null cells exhibit poorer survival in a clonogenic assay (Fig. 4, Appendix I). Additionally, although we were unable initially to maintain the Her-2/Neu-expressing H16N2 cells, we have now established those cells for subsequent experiments relating Her-2/Neu expression with Bcl-3 activity. We have now received the Bcl-3 null mice which will allow us to begin our animal experimentation.

KEY RESEARCH ACCOMPLISHMENTS:

-Generation of an antibody that recognizes mouse Bcl-3 (required for our animal studies proposed for years 2 and 3).

-Obtaining the Bcl-3 null animals for future experiments.
-Generation of Bcl-3 expressing MCF-7 breast cancer cell lines for studies on Bcl-3-dependent gene expression and control of chemoresistance.
-Identification of a role for Bcl-3 in controlling p53 tumor suppressor function (Appendix I, Figures 1-4). These studies indicate that Bcl-3 suppresses apoptosis potentially through the ability to block p53 induction. The may be explained through the ability of Bcl-3 to control Mdm2 gene expression. These studies provide new and important insight into the regulatory functions of Bcl-3, potentially explaining an oncogenic function of Bcl-3 related to modulation of p53 function through control of Mdm2 activity.

REPORTABLE OUTCOMES:
We will be preparing very soon a manuscript describing the requirement of Bcl-3 to control p53 function (see Key Research Accomplishments described above).

CONCLUSIONS:
Bcl-3, which is described as an oncoprotein in certain leukemias and lymphomas, is expressed in breast cancer animal models and in human breast tumors. Our present data indicate the very interesting possibility that Bcl-3 controls p53 function in a negative manner while functioning to promote cell proliferation through upregulation of Cyclin D1 (often overexpressed in breast cancer). The data may explain that breast tumors with Bcl-3 expression are actually inhibited for the tumor suppressor function of p53 via the potential upregulation of Mdm2 or some other regulatory pathway controlling p53. The data may also explain why Bcl-3-expressing tumors are chemo- or radioresistant.

REFERENCES:

Cogswell, P.C., D. Guttridge, W. Funkhouser, and A. Baldwin. 2000. Selective activation of NF-kB subunits in human breast cancer: potential roles for NF-kB2/p52 and Bcl-3. Oncogene 19, 1123-1131.

Hu, M.C., D.F. Lee, W. Xia, L.Golfman, F. Yang, J. Yang, Y. Zou, S. Bao, N. Hanada, H. Saso, R. Kobayashi, and M.C. Hung. 2004. IKK promotes tumorigenesis through inhibition of Forkhead Foxo3a. Cell 117, 225-237.

Pratt, M.A., T. Bishop, D. White, G. Yasvinski, M. Menard, M. Niu, and R. Clarke. 2003. Estrogen withdrawal-induced NF-kB activity and Bcl-3 expression in breast cancer cells: roles in growth and hormone independence. Mol. Cell. Biol. 23, 6887-6900.

Romieu-Mourez, R., D. Kim, S.Shin, E. Demico, D. Landesman-Bollag, D. Seldin, R. Cardiff, and G. Sonenshein. 2003. Mouse mammary tumor virus c-rel transgenic mice develop breast tumors. Mol. Cell. Biol. 23, 5738-5754.

Westerheide, S., M. Mayo, V. Anest, J. Hanson, and A. Baldwin. 2001. The putative oncoprotein Bcl-3 induces cyclin D1 to induce G1 transition. Mol. Cell. Biol. 21, 8428-8436.

Appendix I

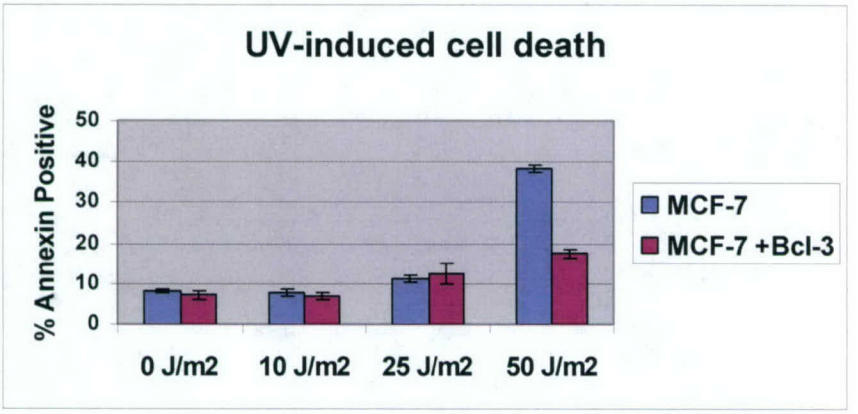

Figure 1. Cells over-expressing Bcl-3 are protected against UV-induced apoptosis. Parental and Bcl-3 Over-expressing MCF-7 cells were either treated with 0, 10, 25 or 50J/m^2 UV-C and harvested 24 hours later. Cells were then washed in PBS and stained with Alexa 488 conjugated Annexin-V (Molecular Probes) and propidium iodide (Sigma). Annexin-V staining levels were measured by flow cytometry (Facscalibur, Becton Dickinson)

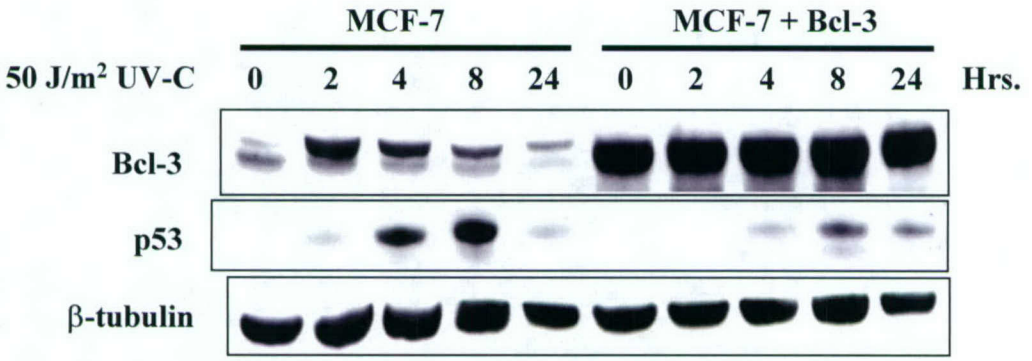

Figure 2. p53 induction is compromised in cells over-expressing Bcl-3. Parental and Bcl-3 Over-expressing MCF-7 cells were treated with 50J/m^2 UV-C and harvested at the indicated timepoints. Cells were washed in PBS, lysed in RIPA buffer and 10ug of each sample was run on 10-20% polyacrylamide gel. The gel was transferred to a nitrocellulose membrane and probed with antibodies against Bcl-3 (Upstate Biotech), p53 (Santa Cruz) and β-tubulin (Santa Cruz).

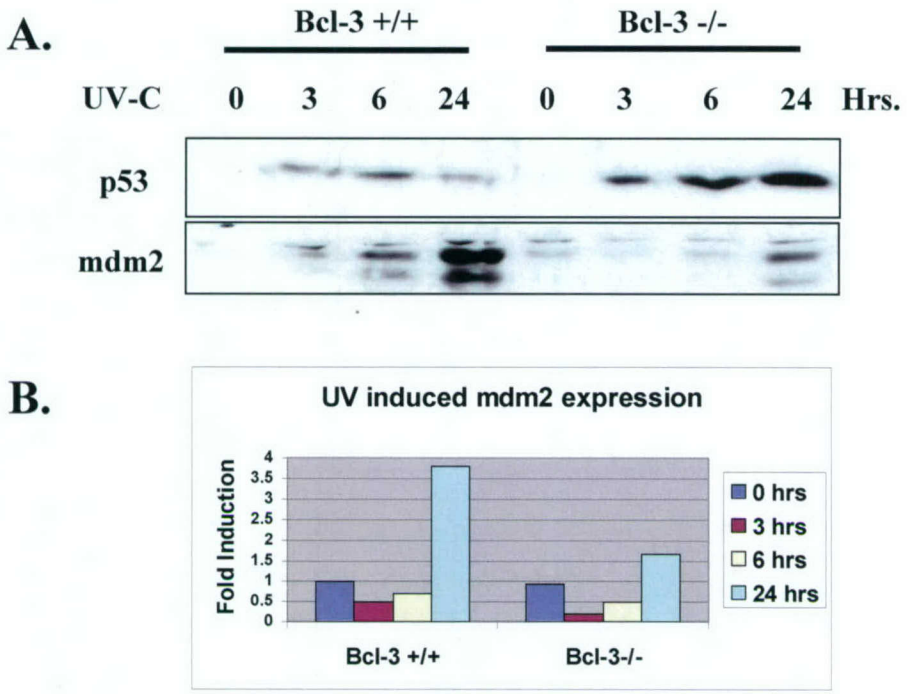

Figure 3. <u>UV-inducued mdm-2 expression is decreased in Bcl-3 deficient fibroblasts</u>. **Wild-type and Bcl-3 deficient mouse embryonic fibroblasts were treated with 50J/m^2 UV-C and harvested at the indicated timepoints. A. Cells were washed in PBS, lysed in RIPA buffer and 10ug of each sample was run on 10-20% polyacrylamide gel. The gel was transferred to a nitrocellulose membrane and probed with antibodies against p53 (Santa Cruz) or mdm-2 (from A. Levine). B. RNA was isolated with trizol reagent (invitrogen), cDNA was made using random primers (invitrogen) and real time PCR was performed (ABI 7000) using primers specific for mouse mdm2 (ABI).**

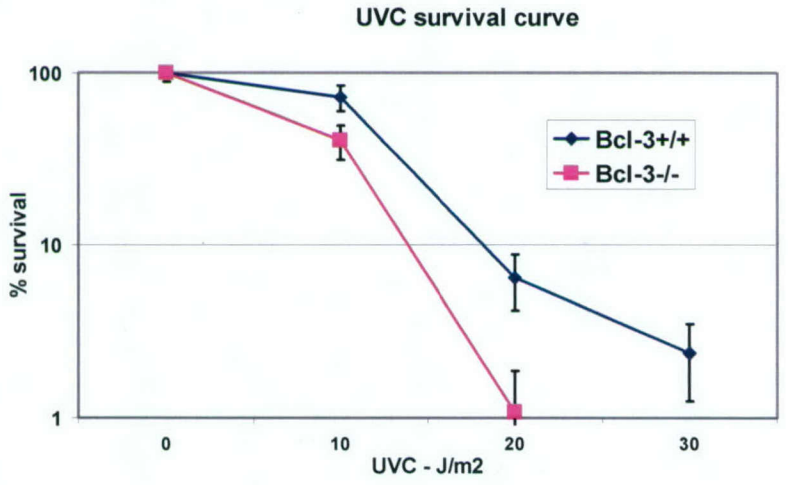

Figure 4. <u>Bcl-3 deficient fibroblasts exhibit decreased clonogenic survival following UV treatment</u>. **Wild-type and Bcl-3 deficient mouse embryonic fibroblasts were plated at 10^3 cells per 100mm dish and treated with the indicated doses of UV-C. One week following treatments the plates were washed and stained with crystal violet (Sigma). Colonies were counted and % survival was determined as the ratio of colonies in UV-treated versus untreated plates.**